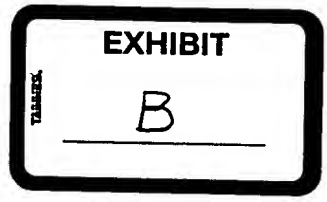

Angiogenesis (1)

1: J Formos Med Assoc. 2000 Aug;99(8):603-11.

Effects of VEGF121 and/or VEGF165 gene transfection on collateral circulation development.

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BACKGROUND AND PURPOSE: Angiogenesis is regulated by various factors, including vascular endothelial growth factor (VEGF). Five isoforms of VEGF have been discovered: VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206. The teleologic basis for the various VEGF isoforms remains unclear, but different VEGF isoforms may mediate distinct endothelial cell functions such as angiogenesis, vascular permeability, and differentiation. We sought to determine the effects of various VEGF isoforms on angiogenesis under ischemic conditions in rabbits. METHODS: The effects of VEGF121 and/or VEGF165 gene transfection on collateral circulation development in ischemic rabbit hindlimb muscles were investigated by using naked plasmids encoding VEGF121 or VEGF165 (pVEGF121 or pVEGF165), either individually or in combination. pCMV beta was used as the control plasmid. The femoral artery on one side of New Zealand White rabbits was ligated. Ten days later, the ischemic muscles received direct intramuscular injection of pVEGF121 (500 micrograms), pVEGF165 (500 micrograms), or pVEGF121 (250 micrograms) + pVEGF165 (250 micrograms) in experimental groups, while pCMV beta (500 micrograms) was used in the control group. Therapeutic effects were evaluated 30 days later by anatomic and physiologic analysis. RESULTS: Internal iliac angiography showed strong development of collateral circulation in all of the pVEGF-treated groups. In contrast, collateral arteries developed weakly in the control group. Combination treatment with both pVEGF121 and pVEGF165 did not result in additional improvement compared with pVEGF121 or pVEGF165 treatment alone (angiographic scores: pVEGF121 = 0.85 +/- 0.10; pVEGF165 = 0.81 +/- 0.11; pVEGF121 + pVEGF165 = 0.83 +/- 0.09; control = 0.53 +/- 0.09; $p < 0.01$). A favorable response in the development of circulation at the capillary level with pVEGF121 and/or pVEGF165 versus pCMV beta was also found. Blood pressure measurement and regional blood flow measurement using colored microspheres revealed similar results. CONCLUSIONS: Our results show that direct intramuscular injection of naked DNA encoding VEGF121 or VEGF165, individually or in combination, is an effective method for gene transfer in an animal model of ischemic limbs and results in augmented collateral vascular development and tissue perfusion.

PMID: 10969502 [PubMed - indexed for MEDLINE]

1: Proc Natl Acad Sci U S A. 1997 Oct 28;94(22):12081-7. (2)

Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF121 and VEGF165 but not VEGF189.

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The vascular endothelial growth factor (VEGF) has been shown to be a significant mediator of angiogenesis during a variety of normal and pathological processes, including tumor development. Human U87MG glioblastoma cells express the three VEGF isoforms: VEGF121, VEGF165, and VEGF189. Here, we have investigated whether these three isoforms have distinct roles in glioblastoma angiogenesis. Clones that overexpressed each isoform were derived and inoculated into mouse brains. Mice that received VEGF121- and VEGF165-overexpressing cells developed intracerebral hemorrhages after 60-90 hr. In contrast, mice implanted with VEGF189-overexpressing cells had only slightly larger tumors than those caused by parental cells and little evidence of hemorrhage at these early times after implantation, whereas, after longer periods of growth, enhanced angiogenicity and tumorigenicity were apparent. There was rapid blood vessel growth and breakdown around the tumors caused by cells overexpressing VEGF121 and VEGF165, whereas there was similar vascularization but no eruption in the vicinity of those tumors caused by cells overexpressing VEGF189, and none on the border of the tumors caused by the parental cells. Thus, by introducing VEGF-overexpressing glioblastoma cells into the brain, we have established a reproducible and predictable in vivo model of tumor-associated intracerebral hemorrhage caused by the enhanced expression of single molecular species. Such a model should be useful for uncovering the role of VEGF isoforms in the mechanisms of angiogenesis and for investigating intracerebral hemorrhage due to ischemic stroke or congenital malformations.

PMID: 9342366 [PubMed - indexed for MEDLINE]

(3)

Mol Endocrinol. 1991 Dec;5(12):1806-14.

The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA.

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Vascular endothelial growth factor (VEGF) was recently identified as a secreted, direct-acting mitogen specific for vascular endothelial cells and capable of stimulating angiogenesis in vivo. Molecular cloning revealed multiple forms of VEGF, apparently arising from alternative splicing of its RNA transcript. We have examined various human cDNA libraries by the polymerase chain reaction technique and discovered a fourth molecular form, VEGF206. This form contains a 41-amino acid insertion relative to the most abundant form, VEGF165, and includes the highly basic 24-amino acid insertion found in VEGF189. Southern blot analysis revealed that a single gene encoded these various forms, and nucleic acid sequence analysis of a portion of the VEGF gene revealed an intron/exon structure compatible with alternative splicing of RNA as a mechanism for their generation. Transient transfection of human embryonic kidney 293 cells showed that, like VEGF189, VEGF206 was predominately cell-associated and only very poorly secreted despite the presence of the signal peptide identical to that found in VEGF121 and VEGF165, both of which are efficiently exported from the cell. Vascular permeability activity was detected in the medium of 293 cells transfected with all four forms of VEGF; [redacted]. Thus, alternative splicing of VEGF RNA can produce four polypeptides with strikingly different secretion patterns, which suggests multiple physiological roles for this family of proteins.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors	**Liat MINTZ, Kinneret SAVITZKY and Sharon ENGEL**
Serial No.	**09/884,050**
Filed	20/JUN/2001
Title	**Variable vegf/novel nucleic acid and amino acid sequences**
Group Art Unit:	1646
Examiner:	JIANG, DONG

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION

(1) SIR:

We, Zoya Gluzman-Poltorak and Belly Koren, do hereby declare:

1. I, Zoya Gluzman-Poltorak, hold a Ph. D. degree from the Dept. of Biology, Technion – Israel Institute of Technology, Haifa, Israel. The subject of my thesis is characterization of Neuropilin-2 – a novel VEGF receptor. The subject of my M. Sc. thesis is characterization of VEGF145- a novel VEGF splice variant.

   Since the year 2000, I have been working at the Molecular Cardiology Laboratory, Carmel Medical Center, Haifa, Israel, as a Project Manager.

   I am experienced in the following fields: state-of-the art molecular biology techniques, construction of recombinant mammalian and insect expression systems and tissue culture techniques; DNA and RNA techniques; protein expression, purification and analysis; cell biology and signal transduction research; polyclonal antibodies production and purification; histology techniques including immunostaining and in-situ hybridization; adeno- and retroviral vector construction, production and purification for human gene therapy applications; animal models establishment (pig, sheep, mouse); preclinical trials conductance; and regulatory processes. A copy of my Curriculum Vitae is attached.

2. I, Belly Koren, have am M. Sc. degree from The Faculty of Food Engineering and Biotechnology, Technion - Israel Institute of Technology. I work at the Molecular Cardiology laboratory, Carmel Medical Center, Haifa, Israel. A copy of my Curriculum Vitae is attached.

3. The aim of the experiments described below was to investigate the properties of $VEGF_{114}$ - a novel VEGF isoform. In order to characterize $VEGF_{114}$ it was over-expressed as a protein in endothelial cells (EC) and smooth muscle cells (SMC) using adeno- and retroviral expression systems and its biological properties were compared with those of the known $VEGF_{165}$ isoform.

METHODS

I. Generation of recombinant viral vectors:

Generation of recombinant Adenoviral vectors encoding *$VEGF_{114}$* gene

The recombinant adenoviral vector expressing the human $VEGF_{114}$ gene was constructed by a modified AdEasy protocol (Vogelstein B. PNAS 1998). A 426bp BamHI-XhoI fragment of $VEGF_{114}$ cDNA was inserted into the BglII- XhoI sites in the pAdShuttle-CMV vector under the control of the CMV promoter. The shuttle vectors were linearized by PmeI digestion and purified by Qiaquick gel extraction kit. The linearized shuttle vector and pAdEasy-1 were co-transformed into competent BJ5183 cells by electroporation. Positive clones containing the recombinant adenoviral vectors were selected according to PCR and restriction map analysis. The recombinant adenoviral plasmids were linearized by PacI digestion, purified and transfected into 293 cells using Lipofectamine 2000 (Gibco BRL, USA). Seven days after transfection CPE (cytopatic effect) occurred and 100% of the cells expressed Green Fluorescent Protein (GFP). The cells were harvested and viral extracts were further amplified in 293 cells. The viral stock titer was determined by serial dilution assay in 293 cells and was ~10^{11} pfu/ml. The expression of the transgene was confirmed by Western analysis of conditioned media from the infected cells.

Construction of retroviral vectors for expression of *$VEGF_{114}$* or co-expression of *$VEGF_{114}$* and GFP

The recombinant retroviral vector LXSN-VEGF encoding the human VEGF gene was constructed by inserting the human $VEGF_{114}$ cDNA 426bp EcoRI-XhoI fragment into the EcoRI-XhoI site of plasmid pLXSN (# K1060-B Clontech, USA) under the control of Mo-MULV 5' long terminal repeat (LTR).

The recombinant retroviral vectors expressing the human $VEGF_{114}$ and /or the EGFP genes were constructed by cloning into pLXSN plasmid in two steps. First, an IRES-EGFP EcoRI-HpaI fragment (1400 bp) excised from pIRES2-EGFP (#6029-1 Clontech) was inserted into EcoRI-HpaI sites in pLXSN for construction of the control plasmid, pLXSN-IRES-EGFP. The second step was construction of pLXSN-VEGF114-IRES-EGFP by cloning of a human VEGF114 EcoRI-Xho I fragment (426 bp) into the EcoRI-Sal I sites in pLXSN-IRES-EGFP. The expression cassettes are regulated by the Mo-MULV 5' long terminal repeat (LTR).

Generation of pseudotyped recombinant retroviral vectors encoding *$VEGF_{114}$*

For retroviral vector production, pLXSN-$VEGF_{114}$-IRES-GFP vector or pLXSN-$VEGF_{114}$ was transfected into 293FLYA packaging cells using Lipofectamine (Gibco BRL, USA). After 48 hours, supernatant from confluent cultures of viral producer cells was collected, filtered (0.45 μm) and added to 293 FLYGALV packaging cells. Transduced cells were grown under G418 selection (400 μg/ml) and individual colonies were collected and screened for EGFP

expression, using an inverted fluorescent microscope, and $VEGF_{114}$ expression by Western analysis of transduced cell-conditioned medium. The viral titer of each colony was determined via transduction of TE671 cells and the titers of ~10^6 pfu/ml were obtained. The colonies with the highest-titers were selected and supernatant was collected freshly for transduction of EC and SMC.

II. Verifying transgene expression after gene transfer

Cell culture

Endothelial cells (EC) were isolated from human saphenous veins (HSVEC), and cultured on gelatin-coated dishes in M20 containing M-199 Medium (Biological Industries, Israel) supplemented with 20% FCS, 2mM L-Glutamin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, 100μg/ml Heparin (Sigma) and 2ng/ml bFGF (Enco). Human EC were identified by immunohistochemistry analysis with anti Von-Willebrand factor specific antibodies (Zymed, USA). Smooth muscle cells (SMC) were cultured by explant outgrowth from human saphenous veins (HSVSMC) and left internal mammary arteries (HLSMC). Cells were cultured in Dulbeco's Modified Eagles Medium (DMEM) (Biological Industries, Israel) supplemented with 10% human serum, 2 mM L-Glutamin, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 2ng/ml bFGF. SMC were identified by immunohistochemistry analysis using specific anti-α smooth muscle actin antibodies (Zymed, USA).

The packaging cell lines 293-FLYA, 293-FLYGALV and TEFLYGA (obtained from Dr F.L. Cosset - Lyon, France) were grown in DMEM supplemented with 10% FCS, 2mM L-Glutamine, 100 units/ml penicillin, 0.1mg/ml streptomycin, 6μg/ml blasticidin and 6μg/ml phleomicin.

Infection of EC and SMC with recombinant adenovirus vectors

Endothelial cells and smooth muscle cells were infected with recombinant adenoviral vectors as follows: the cells were seeded at 70% confluence on fibronectin pre-coated plates (4.5μg/ml) 20 hours prior to infection and grown in complete medium (M20). At the day of infection the medium was replaced with fresh M199 (without serum) and the recombinant virus was added at Multiplicity of Infection of 1000 (MOI=1000). The cells were incubated for 90 minutes with gentle tilt every 20 minutes. After infection the virus-containing medium was replaced with complete medium (M20).

Transduction of EC and SMC with recombinant retroviral vectors

Transduction of endothelial cells and smooth muscle cells with the retroviral vectors was performed as followed: EC (passage 4-9) were seeded (10^5 cells per 35-mm well) in fibronectin-coated plates (4.5μg/ml) and grown in complete medium for 24 hours. One hour prior to transduction, the medium was replaced with serum free M199 containing 0.1mg/ml of the cationic polymer DEAE-dextran (Sigma). After pre-conditioning, the cells were washed three times with phosphate-buffered saline (PBS). Transduction was performed by incubation of the cells for 4 hours with supernatants containing viruses freshly collected and filtered (0.45μ) from the virus-producing packaging cell lines. At the end of the incubation the medium was replaced with fresh M20 medium.

III. $VEGF_{114}$ over-expression by infected EC and SMC

Western blot analysis

$VEGF_{114}$ protein expression by adenoviral- or retroviral-infected EC and SMC was detected by ELISA or Western blot analysis of the conditioned medium. 24 hours post infection the medium was changed to serum free medium and cells were grown for an additional 48 hours.

For Western blot analysis the samples of the conditioned medium (30µl) were separated on 8% SDS polyacrylamide gel under reducing conditions, and electrotransferred to nitrocellulose membrane (Shleicher & Schuell). The blots were blocked with 0.1% skim milk in TBS containing 0.3% tween-20 (TBST) for 1 hour at room temperature using gentle agitation. The blots were incubated with primary antibody diluted in blocking solution for 2 hours at room temperature. 1:500 dilution of polyclonal rabbit anti-VEGF antibody (#SC 152 Santa- Cruz, USA) was used for $VEGF_{114}$ detection. Following the incubation the blots were washed three times with TBST and incubated for 1 hour at room temperature with anti rabbit peroxidase-conjugate antibody (Sigma) diluted 1:7000 in TBST. After three washes with TBST, bound antibody was visualized using ECL reagents (Sigma) and exposed to X-ray film.

To detect $VEGF_{114}$ protein concentration in the conditioned medium of viral infected cells, a Human VEGF ELISA kit (Oncogene, Cat# QIA51) was used according to the manufacturer's instructions.

IV. Physiological Effects of *$VEGF_{114}$* gene transfer

Proliferation assay with conditioned medium of rAd *$VEGF_{114}$* infected EC

Conditioned medium containing $VEGF_{114}$ was collected as follows: EC (passages 5-11) were seeded in 6-well plates at 150,000 cells/well and infected with rAd $VEGF_{114}$, rAd $VEGF_{165}$–GFP or rAdGFP. Non-infected cells were used as an additional control group.. 24 hrs after infection the medium was changed to serum free medium and the cells were grown for an additional 48 hours. The conditioned medium was collected and used for the following proliferation assay.

To perform the proliferation assay, EC were seeded at $2x10^4$ cells/ well in 24 well plates pre-coated with fibronectin (4.5µg/ml) in M199 containing 5% FCS. The assay was performed in triplicates. Increasing volumes of conditioned medium containing $VEGF_{114}$, VEGF or control were added each other day. Proliferation rate was detected 6-7 days after infection by cell counting.

Proliferation assay with recombinant retro *$VEGF_{114}$* transduced EC

EC (passages 5-11) were tranduced with recombinant retro $VEGF_{114}$–GFP, $VEGF_{165}$–GFP or GFP viruses. Non-infected cells served as an additional control group. Retrovirus transduced cells were isolated by selection with 250 µg/ml of G418. After selection the cells were seeded at the concentration $6x10^5$ cells/well in 6-well plates pre-coated with fibronectin (4.5µg/ml) in M199 containing 20% FCS. The assay was performed in duplicate. Proliferation rate was detected 7 and 10 days after seeding by cell counting.

In-vitro angiogenesis assay – sprouting assay in collagen

In-vitro angiogenesis was examined using endothelial cell (EC), smooth muscle cell (SMC), and mixed EC and SMC coculture sprouting from spheroids in collagen three-dimensional matrix. The generation of spheroids was performed as described by Korff T. and Augustin H. (JBC 1998). EC were tagged with DiI-291 red fluorescent marker prior to mixing. 750 cells (EC or SMC) per single culture spheroid or 375 cells from each type (EC and SMC) for coculture spheroid were suspended in culture medium containing 0.25% (w/v) carboxymethylcellulose, seeded in nonadherent round-bottom 96-well plates (Nunc, Denmark). During 24 h incubation at 37°C, 5% CO_2 the suspended cells form a single spheroid per well of defined size and cell number. The spheroids generated were then embedded in collagen gels. A collagen stock solution was prepared prior to use by mixing 8 vol acidic collagen extract of rat tails (equilibrated to 2 mg/ml, 4°C) with 1 vol 10 x M199 (Gibco BRL, USA); 1 vol neutralization solution containing 0.34 N NaOH and 7.5% $NaHCO_3$ to adjust the pH to 7.4. This stock solution (0.5 ml) was mixed with 0.5 ml room temperature medium M199 with 40% human serum containing 0.5% (w/v) carboxymethylcellulose to prevent sedimentation of spheroids before polymerization of the collagen gel. The spheroids (20-30) containing gel was rapidly transferred into prewarmed 24-well plates and allowed to polymerize. The gels were incubated at 37°C, 5% CO_2 and documented using a digital video camera (DXM1200 Nikon, Japan).

Apoptosis assay

The percentage of cells within a population that are actively undergoing apoptosis may be determined by assessing the binding of Annexin V-PE to phospholipid phosphatidylserine (PS). The assay relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS, and binds to cells with exposed PS. Annexin V maybe conjugated to fluorochromes such as Phycoerythrin (PE). This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometry analysis of cells that are undergoing apoptosis. 7-Amino-actinomycin (7-AAD) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. Cells that stain positive for Annexin V-PE and negative for 7-AAD are undergoing apoptosis. Cells that stain positive for both Annexin V-PE and 7-AAD are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-PE and 7-AAD are alive and not undergoing measurable apoptosis.

Retroviral vector transduced HSVEC were seeded onto gelatinized 6-well plates ($2*10^5$ cells/well) in M199 medium supplemented with 20% (v/v) fetal calf serum (FCS) and incubated for 24 h.

In order to induce apoptosis, the cells were "pulse" exposed to H_2O_2: the medium was changed to 1ml of modified-PBS + 1507M H_2O_2 for 1 hour at 37^0 and then changed to M199 medium supplied with 2% FCS and incubated for 24 h.

All floating cells and all adherent cells were collected to the same tube after trypsinization (0.05% trypsin-EDTA). The cells were then pelleted, washed with ice-cold PBS, and resuspended in 1x binding buffer at a concentration of $1*10^6$ cells/ml.

Annexin V-PE and 7-AAD (ready-made solutions) were added to the cells, mixed and incubated for 15 minutes at RT in the dark. Finally, 1x binding buffer was added to a final volume of 0.5ml. Analysis by a FACScan flow cytometer equipped with a doublet discriminating module was performed within 1 hour.

For each checked cell we used two controls. One of unstained cells and the other of Annexin V stained alone, in order to reduce the GFP signal that interferes with the PE signal.

RESULTS

Sequence analysis of $VEGF_{114}$ cDNA

Both strands of the $VEGF_{114}$ cDNA were sequenced and the nucleotide sequence was compared with published VEGF sequence. $VEGF_{114}$ cDNA consists of exons 1-5 which contain information required for the recognition of the known VEGF receptors VEGFR1 and VEGFR2 and are present in all VEGF isoforms (Keyt, B.A., 1997). The $VEGF_{114}$ cDNA lacks exons 6 and 7 encoding heparin binding domains and present in $VEGF_{145}$ and $VEGF_{165}$ respectively. $VEGF_{189}$ contains both of the heparin binding domains. The $VEGF_{114}$ cDNA also lacks exon 8 which exists in all other known VEGF isoforms. To conclude, $VEGF_{114}$ cDNA resembles the structure of $VEGF_{121}$ cDNA without exon 8. We have not found any mutation in the $VEGF_{114}$ cDNA.

$VEGF_{114}$ expression in EC and SMC

To study the properties of $VEGF_{114}$, $VEGF_{114}$ cDNA was expressed in EC and SMC using adenoviral and retroviral expression systems. Transgene expression was verified by detection of fluorescence from GFP. Transduction of EC and SMC by retroviral vector encoding $VEGF_{114}$-GFP demonstrated the production of the GFP protein (figure 1).

$VEGF_{114}$ protein expression was examined by Western blot analysis (~18 kD protein corresponding to the $VEGF_{114}$ protein) after infection of EC with an adenoviral vector (figure 2) or retroviral vector (figure 3) encoding $VEGF_{114}$.To detect $VEGF_{114}$ protein concentration in the conditioned medium of infected cells we used a Human VEGF ELISA kit (Oncogene, Cat# QIA51). The concentrations of $VEGF_{114}$ and $VEGF_{165}$ proteins were 2.7ng/µl and 0.3ng/µl respectively.

$VEGF_{114}$ biological activity

The biological activity of the recombinant $VEGF_{114}$ was tested using a proliferation assay, an in-vitro angiogenesis assay and an apoptosis assay.

The proliferation assays were performed with 1) naive EC by addition of conditioned medium from rAd $VEGF_{114}$ infected EC or 2) with recombinant retro$VEGF_{114}$ transduced EC. In both kinds of experiments $VEGF_{114}$ induces proliferation of EC, but it was less potent than $VEGF_{165}$ (figures 4 and 5).

The effects of $VEGF_{114}$ expression was tested on EC sprouting from spheroids in collagen three-dimensional matrix. Endothelial cells over expressing $VEGF_{114}$ after retroviral gene transfer had no effect on EC sprouting while EC over expressing $VEGF_{165}$ exhibited an increased sprouting (figure 6A). The same results were obtained from co-culture experiments with retrovirus GFP transduced EC and $VEGF_{165}$/GFP or $VEGF_{114}$/GFP retro transduced SMC (figure 6B).

To examine whether over-expression of $VEGF_{114}$ or $VEGF_{165}$ in EC protects the cells from undergoing apoptosis Annexin V-PE staining, which can identify

apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation, was used. FACScan flow cytometer analysis was preformed to detect Annexin V-PE staining. As shown in figure 7, both $VEGF_{114}$ and $VEGF_{165}$ protected EC from apoptosis, though $VEGF_{165}$ was more potent than $VEGF_{114}$.

CONCLUSIONS

From the described study it can be concluded that expression of $VEGF_{114}$ has definite effects on EC proliferation and apoptosis, but it is less potent than $VEGF_{165}$. In addition to the proliferation and apoptosis experiment, a 3-dimentional *in-vitro* angiogenesis assay was performed; $VEGF_{114}$ had no effect on EC sprouting. This $VEGF_{114}$ exhibits two of the three assayed bioactivities of $VEGF_{165}$, although with less potency.

4. The above-described experiments were conducted at the request of Dr. Kinneret Savitsky, who is listed as a co-inventor of the subject matter of the above-identified patent application. The above experiments were performed by Dr. Zoya Gluzman-Poltorak and Mrs.Belly Koren under the supervision of Dr. Moshe Flugelman, who has been informed of the text of the specification and claims of the above-identified patent application.

5. I declare further that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Zoya Gluzman-Poltorak

Belly Koren

Dated this _____th day of October, 2003